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Short Communication

Cdx-2 Homeodomain Protein Expression in Human and Rat Colorectal Adenoma and Carcinoma

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Cancers share many similarities in growth patterns, cellular morphology, and oncofetal antigen expression with embryonic tissue. To better understand the mechanisms underlying malignant transformation and its relationship to developmental processes, we studied the expression of Cdx-2, an intestinal epithelium-specific homeodomain protein, in colorectal adenoma and carcinoma. By immunohistochemistry with a polyclonal Cdx-2 antibody we have shown that Cdx-2 expression is markedly reduced in the later stages of human colorectal carcinogenesis, namely, high grade dysplasia and invasive carcinoma. The same findings occur in 1,2-dimethylhydrazine-induced rat colorectal tumors, confirming the parallels between the rat model and the human disease. As homeodomain proteins play major roles in directing the regionalization of body parts and in organogenesis and cellular phenotypic specification, a reduction of Cdx-2 expression in the late stages of colorectal carcinogenesis may reflect a concomitant deviation of the neoplastic tissue from the normal intestinal epithelial phenotype. (Am J Pathol 1995, 147:586–592)

Colorectal carcinoma develops from stepwise phenotypic alterations of the epithelium as a result of multiple genetic mutations.[1] These mutations affect tumor suppressor genes, oncogenes, and mismatch repair genes, resulting in invasive carcinoma. It is the accumulation of relevant mutations, rather than the order of their occurrence, that is important in the progression to the malignant phenotype. Between individual tumors, there will be variations in the combination of mutated genes, suggesting that colon cancer is a heterogeneous disease. The causes of these mutations are largely unknown although both hereditary and environmental factors are strongly implicated.

Carcinomas frequently display growth patterns and cellular morphology similar to those found in the embryo.[2] Biochemically, they may express antigens that are present in the fetus but are at low or undetectable levels in the adult.[3] It has therefore been argued that the phenotype of a cancer is determined by the developmental stage of the cell from which the malignant clonal expansion occurred.[4] Furthermore, disruption of tissue topographical relationships, ie, its spatial arrangement, can in certain cases give rise to cancer or conversely cause cancers to differentiate to a benign phenotype.[5] As this topographical context is also essential in normal tissue and organ development, Rubin[5] has proposed cancer to be a dynamic developmental disorder. However, if cancer is a disorder of ontogenesis, the mechanisms linking these two processes are unknown.

The homeobox is a highly conserved 180-bp DNA sequence, encoding a 60-amino-acid motif termed the homeodomain. The homeodomain acquires a

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helix-turn-helix structural conformation and is the sequence-specific DNA-binding domain of a family of transcriptional regulatory proteins.[6] Genetic studies have clearly shown that homeobox-containing genes play fundamental developmental roles in determining the regionalization of body parts,[6] organogenesis,[7] and lineage of certain cell types.[8] The *cdx*-2 homeobox gene in the adult mouse is expressed only in the intestinal epithelium.[9] Expression of *cdx*-2 begins in early embryogenesis,[10] increases markedly at mouse embryonic days 17 to 18, and is maintained in the adult.[11] Its transcripts display a gradient of expression along the rostrocaudal axis of the colon, being fivefold greater in the cecum than the rectum. Importantly, Cdx-2 protein expression is not confined to a particular cell lineage, suggesting that it may be responsible for establishing regional, rather than cellular, identity in intestinal epithelium. Furthermore, as its expression is tissue specific and present from the early embryo to the adult, it is likely that *cdx*-2 has a role in both establishing and maintaining the intestinal epithelial phenotype.

In an attempt to better understand the relationship between the expression of developmental genes and malignant transformation, we have used immunohistochemistry to study Cdx-2 protein expression in colorectal tumorigenesis in two settings: human colonic adenomas and carcinomas and 1,2-dimethylhydrazine-induced cancers in rats.

Materials and Methods

Specimen Collection

Specimens of human colonic adenoma and carcinoma were obtained either endoscopically or from surgical resections. For all specimens, histologically normal tissue, within 5 cm of the adenoma or carcinoma, was examined as a control.

DMH-Induced Rat Colonic Carcinoma

Male Sprague-Dawley rats of 100 g weight (4 to 5 weeks old) were treated with 1,2-dimethylhydrazine dihydrochloride (Sigma Chemical Co., St. Louis, MO) as previously described[12] except for the dose, which was reduced from 30 to 20 mg/kg weekly. The rats received weekly subcutaneous 1,2-dimethylhydrazine injections for 10 weeks and were sacrificed 20 weeks after the last injection, at which time 75% of rats had tumors. The colons were removed and flushed with saline, and tumors with adjacent normal tissue were resected and fixed.

Immunohistochemical Procedures

All tissues were processed and analyzed as described.[11] Briefly, tissue was fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) for 1 hour at room temperature, embedded in paraffin wax, and cut into sections (3 μm). Polyclonal antibodies raised in rabbits against a bacterially produced fusion protein containing the amino-terminal 109 amino acids of murine Cdx-2 were then used to detect the protein in these sections. The specificity of the antibody was established both by Western blot analysis[11] and on tissue sections by preincubating the antisera either with 5 μg of purified fusion protein (Cdx-2-maltose-binding protein) or the same amount of bacterial protein (maltose-binding protein). Antigen-antibody complexes were visualized with a peroxidase-based detection system[11] and all sections were counterstained with hematoxylin. Occasionally, a few cells in the lamina propria showed cytoplasmic staining that was not a reproducible finding and is, therefore, likely to be an artifact.

Approval for the study was obtained from the Board of Medical Research and the Ethics Committee on Research of the Royal Melbourne Hospital.

Results

Normal Human and Rat Colonic Epithelium

Cdx-2 was localized to epithelial nuclei, consistent with its role as a transcriptional regulator. Expression in crypt and surface epithelium varied along the rostrocaudal axis of the colon. In humans, from cecum to the splenic flexure, Cdx-2 was strongly expressed in all crypt and surface nuclei. From the descending colon to the rectum, as previously shown,[11] Cdx-2 expression involved mainly the more mature cells, located in an annular cuff of nuclei in the upper one-third of crypts (Figure 1A). In the lower two-thirds of crypts, and on the luminal surface, expression levels were reduced and involved fewer cells. There was no apparent restriction of expression to any particular cell lineage, and even within a given lineage, eg, goblet cells, some nuclei were positive whereas others were not stained, suggesting heterogeneity within cell populations.

The pattern of Cdx-2 expression in the normal tissue of DMH-treated rats (Figure 2A) was identical with that in normal human colon. These findings in human and rat colon are similar to that of the mouse, confirming and extending previous data.[11]

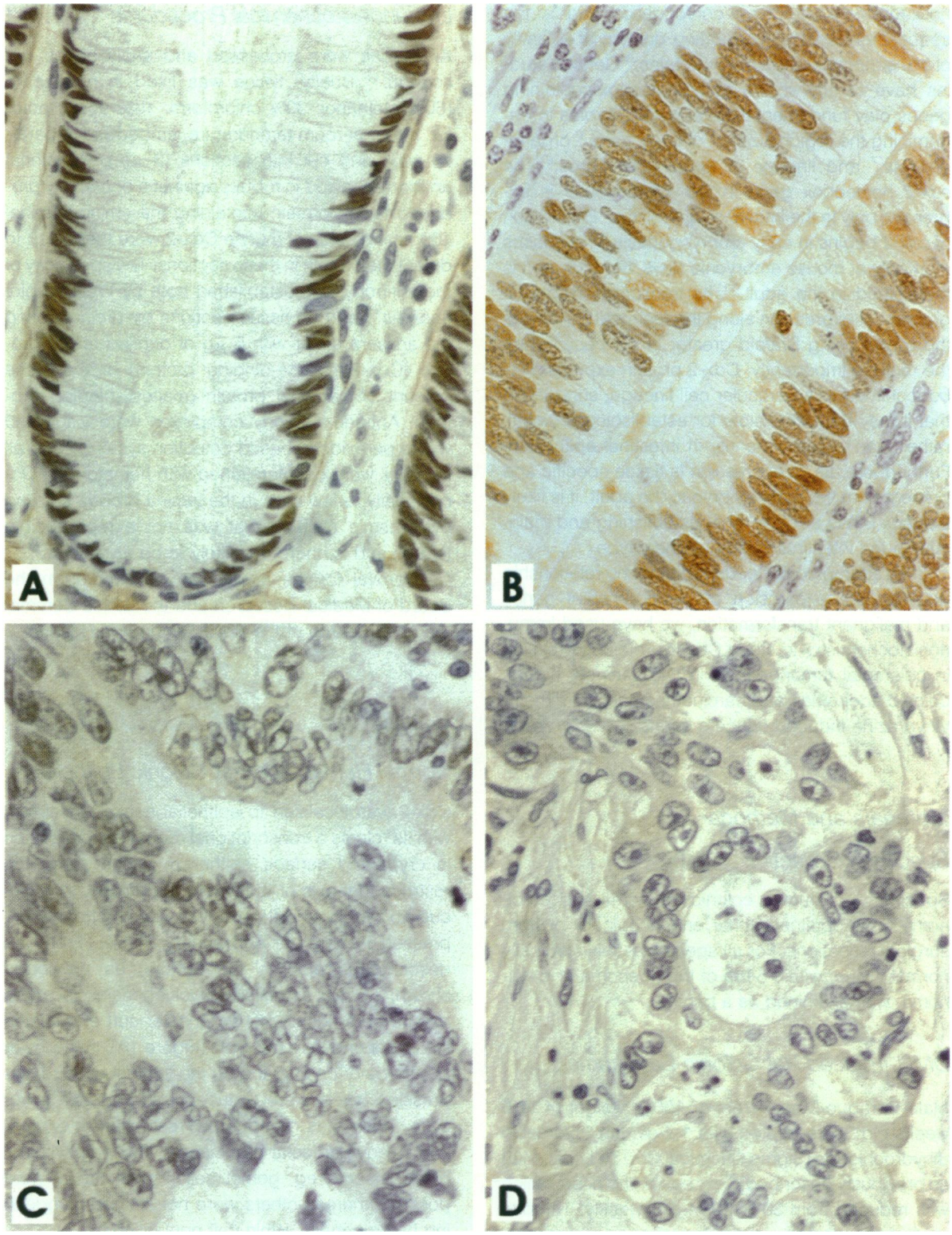

Figure 1. *Immunohistochemical staining of human tissue with polyclonal Cdx-2 antibody.* A: *Basal region of normal rectal crypt.* B: *Low to moderate grade dysplasia, rectal adenoma.* C: *High grade dysplasia, rectosigmoid adenoma.* D: *Invasive carcinoma, sigmoid colon. Magnification,* ×*500.*

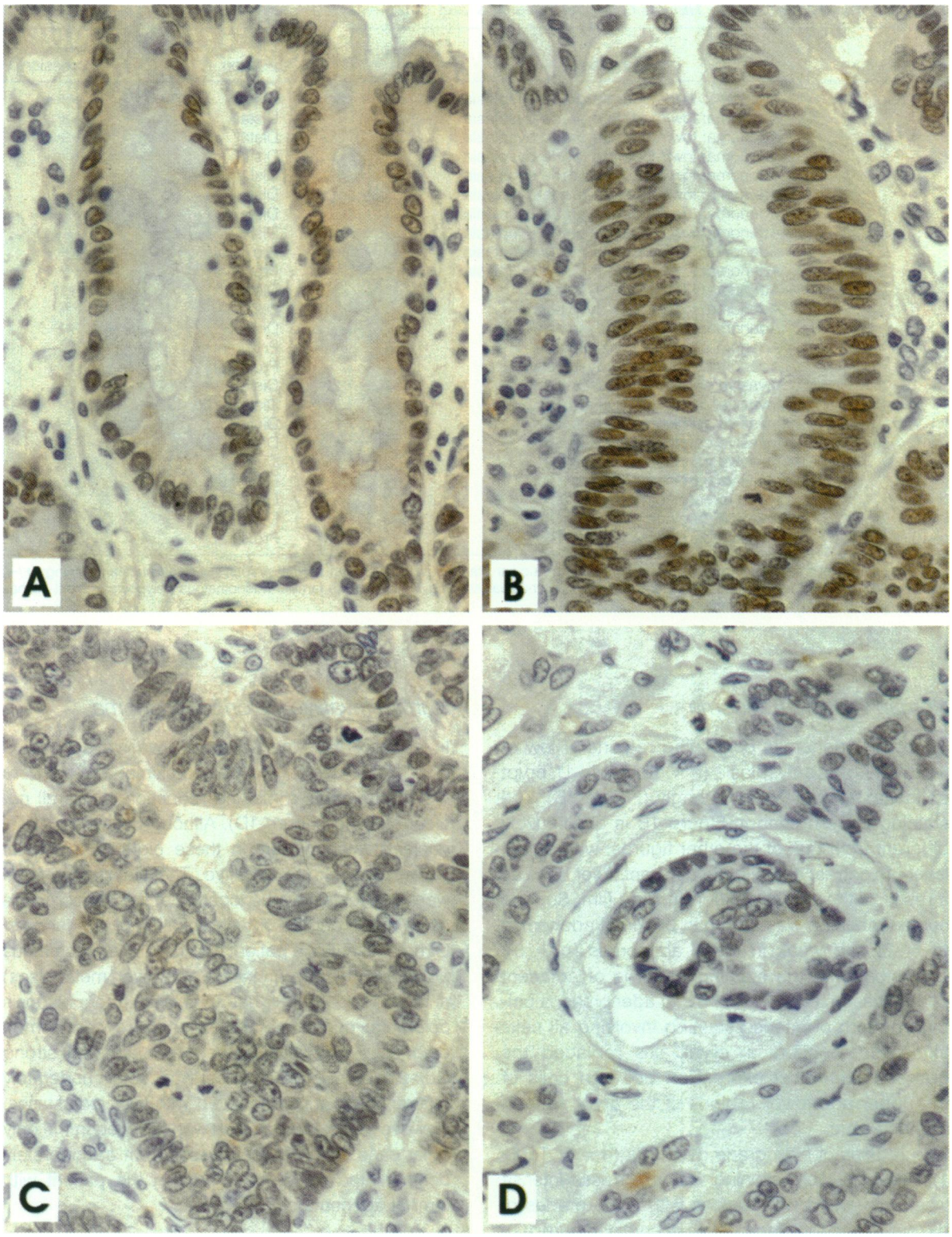

Figure 2. *Immunohistochemical staining of DMH-treated rat tissue with polyclonal Cdx-2 antibody.* A: *Normal colonic crypts, proximal colon.* B: *Low grade dysplasia, upper distal colon.* C: *High grade dysplasia, distal colon.* D: *Invasive carcinoma with lymphatic invasion, distal colon. Magnification, ×500.*

Table 1. *Cdx-2 Expression In Human Colorectal Adenomas and Carcinomas*

Patient	Pathology	Location	Cdx-2 expression
1	Adenoma: low/moderate grade dysplasia	Distal	+++
2	Adenoma: low/moderate grade dysplasia	Distal	+++
4	Adenoma: low/moderate grade dysplasia	Distal	+++
5	Adenoma: low/moderate grade dysplasia	Distal	+++
6	Adenoma: low/moderate grade dysplasia	Distal	++
7	Adenoma: low/moderate grade dysplasia	Distal	+++
8	Adenoma: low/moderate grade dysplasia	Distal	++
9	Adenoma: low/moderate grade dysplasia	Distal	++
14	Adenoma: low/moderate grade dysplasia	Proximal	+++
16	Adenoma: low/moderate grade dysplasia	Proximal	+++
1	Adenoma: high grade dysplasia	Distal	+/−
6	Adenoma: high grade dysplasia	Distal	+
7	Adenoma: high grade dysplasia	Distal	+/−
14	Adenoma: high grade dysplasia	Proximal	+
16	Adenoma: high grade dysplasia	Proximal	+/−
1	Invasive carcinoma (Dukes' D)	Distal	−
3	Invasive carcinoma (Dukes' B)	Distal	−
7	Invasive carcinoma (Dukes' B)	Distal	−
10	Invasive carcinoma (Dukes' B)	Distal	−
11	Invasive carcinoma (Dukes' A)	Distal	−
12	Invasive carcinoma (Dukes' C)	Proximal	+/−
13	Invasive carcinoma (Dukes' C)	Distal	+/−
15	Invasive carcinoma (Dukes' D)	Distal	+++
16	Invasive carcinoma (Dukes' B)	Proximal	−

+++, strong nuclear staining; ++, moderately strong nuclear staining as occurs with normal crypts; +, weak nuclear staining; +/−, weak nuclear staining with some non-staining nuclei; −, no nuclear staining. Patient 1 has familial adenomatous polyposis. Patient 16 has hereditary nonpolyposis colon cancer.

Human Colonic Adenoma

In human adenomas containing regions of low or moderate grade dysplasia (n = 10; Table 1), crypt epithelial nuclei stained strongly (Figure 1B). The staining pattern seen in Figure 1B is qualitatively different from that of the other figures, with a high level of contrast and slightly different color hues as a result of the use of a different slide film. Surface nuclear staining was strong in proximal adenomas (n = 2) and weak in distal adenomas (n = 8), consistent with the staining patterns of adjacent control tissue. However, in distal adenomas, a uniform pattern of intense staining involving all cells along the longitudinal crypt axis replaced the graded, differential expression between cells of the upper and lower regions of the crypt seen in normal tissue.

In regions of high grade dysplasia (n = 5; Table 1), overall nuclear staining was much less intense when compared with regions with lower grades of dysplasia and to adjacent normal crypts (Figure 1C). There was heterogeneity in staining intensity between cells, with some nuclei being weakly stained, whereas others did not stain. We consistently found that cell nuclei displaying the highest degrees of atypia expressed little or no Cdx-2.

Human Invasive Colonic Carcinoma

In eight of the nine cases of invasive carcinoma (Table 1), of which two were proximal and six were distal, there was a marked reduction in overall nuclear staining (Figure 1D). Although some epithelial nuclei stained weakly in these eight cases, most nuclei were Cdx-2 negative. The staining patterns were unaffected by the location of the cancer in the colon or the degree of differentiation of the cancers. Of note is that one patient had familial adenomatous polyposis (rectal carcinoma with liver metastases) and another patient had hereditary nonpolyposis colorectal carcinoma (hepatic flexure carcinoma), diagnosed clinically. In the one case with strong Cdx-2 expression, the cancer was rectal, moderately differentiated and associated with liver metastases. However, Cdx-2 expression was reduced in the only other case with liver metastases from a moderately differentiated rectal carcinoma in the patient with familial adenomatous polyposis.

Dimethylhydrazine Rat Tumors

Low to moderate grade dysplasia was seen in two adenomas from the distal colon, both of which showed staining patterns similar to human adeno-

mas of a comparable grade of dysplasia (Figure 2B). High grade dysplasia was seen in two adenomas (one from proximal and the other from distal colon) and both showed reduced nuclear staining (Figure 2C). Therefore, these observations of adenomas in rats parallel those in humans with comparable grades of dysplasia.

Of the five invasive carcinomas in DMH-treated rats, four arose from distal colon and the other was proximal. The cancers were moderate to poorly differentiated and with one exception (a distal cancer) all showed little or no Cdx-2 expression (Figure 2D). This pattern was similar to that observed in humans. There were no obvious morphological differences between the Cdx-2-positive carcinoma and the remaining carcinomas.

Discussion

We have found that early in human colorectal carcinogenesis, minor changes may occur in Cdx-2 expression, specifically, a loss of the gradient of expression between the upper and lower parts of the crypt normally seen in the distal colon. However, at more advanced stages, namely, in areas of high grade dysplasia, Cdx-2 expression is markedly reduced. In nuclei with the greatest atypia, Cdx-2 is undetectable. There is, therefore, an inverse correlation between the severity of dysplasia and the level of Cdx-2 expression.

Invasive colorectal cancer in humans, like high grade dysplasia, showed little or no Cdx-2 expression in the majority of cases we examined. However, one exception was a rectal cancer, which strongly expressed Cdx-2. This finding confirms that phenotypic differences exist between colon cancers.[13] Interestingly, the cancers arising from patients with either familial adenomatous polyposis (one) or hereditary nonpolyposis colorectal carcinoma (one) both showed reduced Cdx-2 expression. This may suggest that in the stepwise progression of sporadic and inherited colorectal carcinomas, the reduction in Cdx-2 expression occurs beyond the point at which their pathogenetic mechanisms converge. However, examination of additional cases will be required to confirm this finding.

Tumors arising from DMH-treated rats showed similar changes in Cdx-2 expression as in the human disease. As with human cancer, one (of five) did not show decreased Cdx-2 expression, further confirming the heterogeneous nature of the disease. Like the human case, there were no distinguishing morphological features in the Cdx-2-positive carcinoma to explain the difference in immunoreactivity.

Although the suitability of DMH-treated rats as a model of human colorectal carcinogenesis has been questioned,[14] and genetic events in the rat model have not been thoroughly studied, mutations in *ras* oncogenes are found in 66% of rat[15] and 50% of human[1] colorectal cancers. These mutations occur in the early stages of carcinogenesis.[1,15] Using Cdx-2 as another molecular marker, we have shown that its expression in the rat model parallels that in the human disease, confirming their similarities. Importantly, the changes in Cdx-2 expression are observed at a late stage of carcinogenesis, close to the point at which the benign phenotype transforms to become malignant.

A number of studies have attempted to compare homeobox gene expression between solid tumors and their corresponding normal tissues.[16–18] Our study is the only one, to our knowledge, that examines expression of a tissue-specific homeobox gene in neoplasia arising from that tissue. Furthermore, we have been able to precisely identify the cells expressing Cdx-2. Nonhistological methods raise difficulties in interpretation, because of the risk of contamination with nonepithelial tissue. Similarly, *in situ* hybridization with radiolabeled riboprobes does not provide the resolution for cellular localization of gene expression. One other advantage of the present study is that we were able to identify varying degrees of dysplasia within the adenomas and correlate the severity of dysplasia to Cdx-2 expression.

In another attempt to identify differences in gene expression between tumor and normal tissue, a novel cDNA was cloned by subtractive hybridization and predicted to encode a 764-amino-acid open reading frame.[19] The transcript was expressed in normal colonic tissue but not in eight of nine colonic carcinomas nor in six of six colonic adenomas. However, expression of this gene was not proven to be epithelium specific. Interestingly, a partial homeodomain motif spanning 24 amino acids was identified in the predicted protein sequence.

It is in the hemopoietic system that the relationship between homeobox genes and malignancy has been most extensively studied.[20] In certain acute leukemias, chromosomal translocations result in disordered homeobox gene regulation or the formation of chimeric genes by fusion of part of a homeobox gene to another gene.[20] Furthermore, transplanting mouse marrow progenitor cells containing such a chimeric gene (*E2A-PBX1*) into recipient mice resulted in acute myeloid leukemia,[21] and transgenic mice expressing the chimeric gene under the control of an immunoglobulin gene enhancer developed diffuse T cell lymphomas.[22]

Although we have shown an inverse relationship between Cdx-2 immunoreactivity and the severity of dysplasia, a causal effect remains uncertain. This is in contrast to our understanding of homeobox gene involvement in the pathogenesis of certain acute leukemias. It is, therefore, premature to suggest that reduction of Cdx-2 expression levels is, in part, responsible for progression to the more advanced stages of colorectal tumorigenesis. Instead, it may well be that alterations in Cdx-2 expression are part of widespread changes that occur as a consequence of tumor progression. As Cdx-2 expression begins early in embryogenesis and is confined to the intestinal epithelium in the adult mouse, it can be seen as a marker of the normal intestinal epithelial phenotype. Loss of Cdx-2 expression in the late stages of colorectal carcinogenesis may, therefore, indicate a concomitant deviation away from the normal intestinal epithelial phenotype. Importantly, these changes in Cdx-2 expression may, together with additional studies of homeobox genes in neoplasias, provide information establishing a relationship between developmental and neoplastic processes.

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